REMARKS

Applicants submit this amendment to insert required references to the sequence listing into the specification and to provide support of claims 1-18.

Respectfully submitted.

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Version With Markings to Show Changes Made

The gene encoding TPO has been cloned and characterized. See Kuter et al. Proc. Natl. Acad. Sci. USA **91:11104-11108** (1994); Bartley et al. Cell **77:1117-1124** (1994); Kaushansky et al. Nature **369:568-571** (1994); Wendling et al. Nature **369:571-574** (1994); and de Sauvage et al. Nature **369:533-538** (1994). Thrombopoietin is a glycoprotein with at least two forms, with apparent molecular masses of 25 kDa and 31 kDa, with a common N-terminal amino acid sequence. See, Bartley et al. Cell **77:1117-1124** (1994). Thrombopoietin appears to have two distinct regions separated by a potential Arg-Arg cleavage site. The amino-terminal region is highly conserved in man and mouse, and has some homology with erythropoietin and interferon-a and interferon-b. The carboxy-terminal region shows wide species divergence.

The DNA sequences and encoded peptide sequences for human TPO-R (also known as *c-mpl*) have been described. See Vigon et al. Proc. Natl. Acad. Sci. USA **89:5640-5644** (1992). TPO-R is a member of the haematopoietin growth factor receptor family, a family characterized by a common structural design of the extracellular domain, including four conserved C residues in the N-terminal portion and a WSXWS motif **--**(SEQ ID NO:1)**--** close to the transmembrane region. See Bazan Proc. Natl. Acad. Sci. USA **87:6934-6938** (1990). Evidence that this receptor plays a functional role in hematopoiesis includes observations that its expression is restricted to spleen, bone marrow, or fetal liver in mice (see Souyri et al. Cell **63:1137-1147** (1990)) and to megakaryocytes, platelets, and $CD34^+$ cells in humans (see Methia et al. Blood **82:1395-1401** (1993)). Furthermore, exposure of $CD34^+$ cells to synthetic oligonucleotides antisense to mpl RNA significantly inhibits the appearance of megakaryocyte colonies without affecting erythroid or myeloid colony formation. Some workers postulate that the receptor functions as a homodimer, similar to the situation with the receptors for G-CSF and erythropoietin.

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having from 1 to 3 substituents in the phenyl ring selected from the group consisting of lower alkyl, lower alkoxy, chloro, and bromo, where R and R' are independently selected from the group consisting of hydrogen and lower alkyl,

and still further wherein the C-terminus of said peptide or peptide mimetic has the formula -C(=O)-R'' where R'' is selected from the group consisting of hydroxy, lower alkoxy, and $-NR^1R^1$ where R^1 and R^1 are independently selected from the group consisting of hydrogen and lower alkyl and where the nitrogen atom of the $-NR^1R^1$ group can optionally be the amine group of the N-terminus of the peptide so as to form a cyclic peptide,

and physiologically acceptable salts thereof.

In a related embodiment, the invention is directed to a labeled peptide or peptide mimetic comprising a peptide or peptide mimetic described as above having covalently attached thereto a label capable of detection.

In some embodiments of the invention, preferred peptides for use include peptides having a core structure comprising a sequence of amino acids --(SEQ ID NO:2)--:

$X_1\ X_2\ X_3\ X_4\ X_5\ X_6\ X_7$

where X_1 is C, L, M, P, Q, V; X_2 is F, K, L, N, Q, R, S, T or V; X_3 is C, F, I, L, M, R, S, V or W; X_4 is any of the 20 genetically coded L-amino acids; X_5 is A, D, E, G, K, M, Q, R, S, T, V or Y; X_6 is C, F, G, L, M, S, V, W or Y; and X_7 is C, G, I, K, L, M, N, R or V.

In a preferred embodiment the core peptide comprises a sequence of amino acids --(SEQ ID NO:3)--:

$X_1\ G\ X_3\ X_4\ X_5\ X_6\ X_7$

where X_1 is L, M, P, Q, or V; X_3 is F, R, S, or T; X_4 is F, L, V, or W; X_5 is A, K, L, M, R, S, V, or T; X_6 is A, E, G, K, M, Q, R, S, or T; X_7 is C, I, K, L, M or V; and each X_n residue is independently selected from any of the 20 genetically coded L-amino acids, their stereoisomeric D-amino acids; and non-natural amino acids. Preferably, each X_n residue is

L-amino acids and their stereoisomeric L-amino acids. In a preferred embodiment -- SEQ ID NO:4 --, X_1 is P; X_2 is T; X_3 is L; X_4 is R; X_5 is E or Q; and X_6 is I or L.

More preferably, the core peptide comprises a sequence of amino acids -- SEQ ID NO:5 --:

X_1 X_2 G X_3 X_4 X_5 X_6 X_7 W X_8

where X_1 is A, C, E, G, I, L , M, P, R, Q, S, T, or V; and X_2 is A, C, D, E, K, L, Q, R, S, T, or V. More preferably, X_1 is A or I; and X_2 is D, E, or K.

Particularly preferred peptides include --(SEQ ID NOS 6-13, respectively)--: G G C A D G P T L R E W I S F C G G; G N A D G P T L R Q W L E G R R P K N; G G C A D G P T L R E W I S F C G G K; T I K G P T L R Q W L K S R E H T S; S I E G P T L R E W L T S R T P H S; L A I E G P T L R Q W L H G N G R D T; C A D G P T L R E W I S F C; and I E G P T L R Q W L A A R A.

In further embodiments of the invention, preferred peptides for use in this invention include peptides having a core structure comprising a sequence of amino acids --(SEQ ID NO:14)--:

C X_1 X_2 X_3 X_4 X_5 X_6

where X_1 is F, K, L, N, Q, R, S, T or V; X_2 is C, F, I, L, M, R, S or V; X_3 is any of the 20 genetically coded L-amino acids; X_4 is A, D, E, G, S, T or Y; X_5 is D, F, G, L, M, S, V, W or Y; and X_6 is C, G, I, K, L, M, N, R or V. In a more preferred embodiment, X_1 is A, E, G, H, K, L, M, P, Q, R, S, T, or W. In a further embodiment, X_2 is S or T; X_3 is L or R; X_4 is R; X_5 is D, E, or G; X_6 is F, L, or W; and X_7 is I, K, L, R, or V. Particularly preferred peptides include --(SEQ ID NO:15)--: G G C T L R E W L H G G F D G G.

In a further embodiment, preferred peptides for use in this invention include peptides having a structure comprising a sequence of amino acids -- SEQ ID NO:16 --:

X_1 C X_2 X_3 X_4 X_5 X_6 X_7

where X_1 is P, K, L, N, Q, R, S, T or V; X_2 is D, E, I, L, M, R, S, V or W; X_3 is any of the 20 genetically coded L-amino acids; X_4 is A, D, E, G, K, M, Q, R, S, T, V or Y; X_5 is C, F, G, L, M, S, V, W or Y; X_6 is D, G, I, P, L, M, N, R or V; and X_7 is any of the 20 genetically coded L-amino acids. In some embodiments, X_7 is preferably G, S, Y, or R.

The compounds described herein are useful for the prevention and treatment of diseases mediated by TPO, and particularly for treating hematological disorders, including but not limited to, thrombocytopenia resulting from chemotherapy, radiation therapy, or bone marrow transfusions. Thus, the present invention also provides a method for treating wherein a patient having a disorder that is susceptible to treatment with a TPO agonist receives, or is administered, a therapeutically effective dose or amount of a compound of the present invention.

The invention also provides for pharmaceutical compositions comprising one or more of the compounds described herein and a physiologically acceptable carrier. These pharmaceutical compositions can be in a variety of forms including oral dosage forms, as well as inhalable powders and solutions and injectable and infusible solutions.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-B illustrates the results of a functional assay in the presence of various peptides; the assay is described in Example 3. Figure 1A is a graphical depiction of the results of the TPO-R transfected Ba/F3 cell proliferation assay for selected peptides of the invention:

■ designating the results for -- (SEQ ID NO:8) -- G G G A D G P T L R E W I S F G G G K (biotin) ;

X designating the results for -- (SEQ ID NO:8) -- G G G A D G P T L R E W I S F G G G;

designating the results for -- SEQ ID NO:11 -- I A I E G P T L R Q W L H G N G P I T;

designating the results for -- SEQ ID NO:7 -- G N A D G P T L R Q W L E G R R P K N; and

designating the results for -- SEQ ID NO:9 -- T I K G P T L R Q W L K S R E H T S.

Figure 1B is a graphical depiction of the results with the same peptides and the parental cell line.

Figure 2A-C show the results of peptide oligomerization using the TPO-R transfected Ba/F3 cell proliferation assay. Figure 2A shows the results of the assay for the complexed biotinylated peptide (AF 12285 with streptavidin (SA)) for both the transfected and parental cell lines. Figure 2B shows the results of the assay for the free biotinylated peptide (AF 12285) for both the transfected and parental cell lines. Figure 2C shows the results of the assay for streptavidin alone for both the transfected and parental cell lines.

Figures 3A-G show the results of a series of control experiments showing the activity of TPO, the peptides of the present invention, EPO, and EPO-R binding peptides in a cell proliferation assay using either the TPO-R transfected Ba/F3 cell line and its corresponding parental line, or an EPO-dependent cell line. Figure 3A depicts the results for TPO in the cell proliferation assay using the TPO-R transfected Ba/F3 cell line and its corresponding parental line. Figure 3B depicts the results for EPO in the cell proliferation assay using the TPO-R transfected Ba/F3 cell line and its corresponding parental line. Figure 3C depicts the results for complexed biotinylated peptide (AF 12285 with streptavidin (SA)) and a complexed form of a biotinylated EPO-R binding peptide (AF 11505 with SA) in the TPO-R transfected Ba/F3 cell line. The results for the corresponding parental cell line are shown in Figure 3D. Figure 3E depicts the results for TPO in the cell

proliferation assay using the EPO-dependent cell line. Figure 3F depicts the results for EPO in the cell proliferation assay using the EPO-dependent cell line. Figure 3G depicts the results for complexed biotinylated peptide AF 11838 with streptavidin SA and the complexed form of a biotinylated EPO-R binding peptide AF 11838 with SA in the EPO-dependent cell line.

Figures 4A-C illustrates the construction of peptides-on-plasmids libraries in vector pJS142. Figure 4A shows a restriction map and position of the genes. The library plasmid includes the *rrnB* transcriptional terminator, the *bla* gene to permit selection on ampicillin, the M13 phage intragenic region (*M13 IG*) to permit rescue of single-stranded DNA, a plasmid replication origin (*ori*), two $lacO_s$ sequences, and the *araC* gene to permit positive and negative regulation of the *araB* promoter driving expression of the *lac* fusion gene. Figure 4B --(SEQ ID NOS 19 & 20, respectively)-- shows the sequence of the cloning region at the 3' end of the *lac I* gene, including the SfiI and EagI sites used during library construction. Figure 4C --(SEQ ID NOS 223 & 224, respectively)-- shows the ligation of annealed library oligonucleotides, ON-829 and ON-830, to SfiI sites of pJS142 to produce a library. Single spaces in the sequence indicate sites of ligation.

Figures 5A-B illustrate cloning into the pELM3 and pELM15 MBP vectors. Figure 5A --(SEQ ID NOS 225 & 226, respectively)-- shows the sequence at the 3' end of the malE fusion gene, including the MBP coding sequence, the poly asparagine linker, the factor Xa protease cleavagge site, and the available cloning sites. The remaining portions of the vectors are derived from pMALc2 (pELM3) and pMALp2 (pELM15), available from New England Biolabs. Figure 5B --(SEQ ID NOS 227 & 228, respectively)-- shows the sequence of the vectors after transfer of the BspEII-ScaI library fragment into AgeI-ScaI digested pELM3/pELM15. The transferred sequence

includes the sequence encoding the Jil peptide linker from the pJS142 library.

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Figure 6A depicts a restriction map and position of the genes for the construction of headpiece dimer libraries in vector pCMG14. The library plasmid includes: the rrnB transcriptional terminator, the bla gene to permit selection on ampicillin, the M13 phage intragenic region (M13 IG) to permit rescue of single-stranded DNA, a plasmid replication origin (ori), one lacO$_{1}$ ssequence, and the araC gene to permit positive and negative regulation of the araB promoter driving expression of the headpiece dimer fusion gene. Figure 6B --(SEQ ID NOS 229 & 230, respectively)-- depicts the sequence of the cloning region at the 3' end of the headpiece dimer gene, including the SfiI and EagI sites used during library construction. Figure 6C --(SEQ ID NOS 231 & 232, respectively)-- shows the ligation of annealed ON-1679, ON-829, and ON-830 to SfiI sites of pCMG14 to produce a library. Singles spaces in the sequence indicate sites of ligation.

Figures 7 to 9 show the results of further assays evaluating activity of the peptides and peptide mimETICS of the invention. In this assay mice are made thrombocytopenic with carboplatin. Figure 7 depicts typical results when Balb/C mice are treated with carboplatin (125 mg/kg intraperitoneally) on Day 0. The dashed lines represent untreated animals from three experiments. The solid line represent carboplatin-treated groups in three experiments. The heavy solid lines represent historical data. Figure 8 depicts the effect of carboplatin titration on platelet counts in mice treated with the indicated amounts of carboplatin (in mg/kg, intraperitoneally (ip) on Day 0 . Figure 9 depicts amelioration of carboplatin-induced thrombocytopenia on Day 10 by peptide AF12513 (513). Carboplatin (CBP; 80-125 mg/kg, intraperitoneally) was administered on Day 0. AF12513 (1 mg/kg, ip) was given on Days 1-9.

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the consensus sequence mutagenized at [illegible] frequency and extended on each terminus with random residues to produce clones which encode the sequence -- SEQ ID NO:21 -- XXXXX C, S, P, or R TLREWL XXXXXXX D or J . A similar extended/mutagenized library was constructed using the peptides-on-plasmids system to produce clones which encode the sequence -- SEQ ID NO:22 -- XXXXX C, S, P, or R TLREWL XXXXXXX. An additional extended/mutagenized library -- SEQ ID NO:23 --, XXXXX C, S, P, or R TLREWL XXXXXXX D or J, was constructed using the polysome display system. All three libraries were screened with peptide elution and probed with radiolabeled monovalent receptor.

The "peptides on plasmids" techniques was also used for peptide screening and mutagenesis studies and is described in greater detail in U.S. Patent no. 5,338,665, which is incorporated herein by reference for all purposes. According to this approach, random peptides are fused at the C-terminus of LacI through expression from a plasmid vector carrying the fusion gene. Linkage of the LacI-peptide fusion to its encoding DNA occurs via the lacO sequences on the plasmid, forming a stable peptide-LacI-plasmid complex that can be screened by affinity purification (panning) on an immobilized receptor. The plasmids thus isolated can then be reintroduced into *E. coli* by electroporation to amplify the selected population for additional rounds of screening, or for the examination of individual clones.

In addition, random peptide screening and mutagenesis studies were performed using a modified C-terminal Lac-I display system in which display valency was reduced ("headpiece dimer" display system). The libraries were screened and the resulting DNA inserts were cloned as a pool into a maltose binding protein (MBP) vector allowing their expression as a C-terminal fusion protein. Crude cell lysates from randomly picked individual MBP fusion clones were then

assayed for TPO-R binding in an ELISA format, as discussed above.

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Peptide mutagenesis studies were also conducted using the polysome display system, as described in co-pending application U.S. Patent Application Serial No. 08/300,262, filed September 2, 1994, which is a continuation-in-part application based on U.S. Patent Application Serial No. 08/144,776, filed October 29, 1993 and PCT WO 95/11922, each of which is incorporated herein by references for all purposes. A mutagenesis library was constructed based on the sequence -- (SEQ ID NO:14) -- X X X X X (C,F,P,or S) t l r e f l X X X X X X (C or S), in which X represents a random NNK codon, and the lower case letters represent amino acid codons containing 70:10:10:10 mutagenesis at positions 1 and 2 and K (G or T) at position 3 of the codon. The library was panned for 5 rounds against TPO receptor which had been immobilized on magnetic beads. After the fifth round, the PCR amplified pool was cloned into pAFF6 and the ELISA positive clones were sequenced. The sequences were subcloned into an MBP vector and their binding affinities were determined by an MBP ELISA.

To imobilize the TPO-R for polysome screening, Ab 179 was first chemically conjugated to tosyl-activated magnetic beads (available from Dynal Corporation) as described by the manufacturer. The beads were incubated with antibody in 0.5 M borate buffer (pH 9.5) overnight at room temperature. The beads were washed and combined with TPO-R containing the "HPAP" tail. The antibody coated beads and receptor were incubated for 1 hour at 4°C, and the beads were washed again prior to adding the polysome library.

Screening of the various libraries described above yielded the TPO receptor binding peptides shown in Tables 1 and 2 below, as well as others not listed herein.

TABLE 1

-- SEQ ID NOS 25-34, RESPECTIVELY --

Peptide
R E G P T L P Q W M
R E G P T L P Q W M
S R G M T L R E W L
E G P T L R G W L A
R E G Q T L K E W L
E R G P F W A K A C
R E G P R C V M W M
C S G L T L R E W L V C
C L T G P F V T Q W L Y E C
C G E G L T L T Q W L E H C
C R A G P T L L E W L T L C
C R A G P T L L E W L T L C
C R Q G P T L T A W L L E C
C A D G P T L R E W I S F C
C E L V G P S L M S W L T C
C G T E G P T L S T W L D C
C D Q L G V T L S R W L E C
C G T G L T L R E W L G S F S L L S
C P E G P T L L Q W L K R G Y S S C
R G L G P I L S Q W L Y S L M L M C
M V A G P T L R E F I A S L P I H C
S M Q G P T F R E W V S M M K V L C
S V Q C G P T L R Q W L A A R N H L S
G N A D G P T L R Q W L E G R R P K N
S V R C G P T L R Q W L A A R T H L S
L A I E G P T L R Q W L H G N S R D T
H G R V G P T L R E W K T Q Y A T W K
C A D G P T L R E W I S F C

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TABLE 2

-- SEQ ID NOS 89-167, respectively --

Peptide
C S L E D L R K F C
C R R S E L L E R C
C T F K Q F L D G C
C T R G E W L R C C
C T L R Q W L Q G C
C T L E E L R A C C
C T R E E L M R L C
C Q R A D L I N F C
C N F N D L L L F C
C T R T E W L H G C
C T L E F M N G C
C S L G E L R R L C
C N I N Q L R S I C
C T M F Q F L V C C
C T F S E W L E R C
C T I H E Y L S G C
C T R E E L L R Q C
C T F R E F V N G C
C S R A D F L A A C
C S C A Q V V Q C C
C T L R Q W I L L G M C
C T L R E W L H G G F C
C T L R A W L M S E T C
C T L R A W L M E S C C
C T F Q V W K L A R N C
C L L R E W L D K R T C
C V L R E W L L K K S C
C L L S E F L A C Q Q C

synthetic peptides are often preceded by one or two glycine residues. These glycines are not believed to be necessary for binding or activity. Likewise, to mimic the exact sequence of peptides displayed on polysomes, the C-terminal amino acids of the synthetic peptides are often preceded by the sequence M A S. Again, this sequence is not believed to be necessary for binding or activity.

IC_{50} values are indicated symbolically by the symbols "-", "+", and "++". For examples, those peptides which showed IC_{50} values in excess of 200 uM are indicated with a "-". Those peptides which gave IC_{50} values of less than or equal to 200 μM are given a "+", while those which gave IC_{50} values of 500 nm or less are indicated with a "++". Those peptides which gave IC_{50} values at or near the cutoff point for a particular symbol are indicated with a hybrid designator, e.g., "+/-". Those peptides for which IC_{50} values were not determined are listed as "N.D.". The IC_{50} value for peptides having the structure: --(SEQ ID NO:15)-- G G C T L R E W L H G G F C G G was 500 nm or less. (Note the N-terminal and C-terminal amino acids were preceded by two glycines to recreate the exact sequence displayed by the phage. These glycines are not believed to be necessary for binding or activity.)

TABLE 3

--(SEQ ID NOS 6,7,8,9,169,11&10, RESPECTIVELY)--

Peptide	Affinity
G G C A D G P T L R E W I S F C G G	++
G N A D G P T L R Q W L E G R R P H N	++
G G C A D G P T L R E W I S F C G G K	++
T I K G P T L R Q W L K S R E H T S	++
G P T L R Q W L	-
L A I E G P T L R Q W L H G N S R D T	++
S I E G P T L R E W L T S R T P H S	++

The tables above, especially Table 3, illustrate that a preferred core peptide comprises a sequence of amino acids -- SEQ ID NO:2 --:

X_1 X_2 X_3 X_4 X_5 X_6 X_7

where X_1 is C, L, M, P, Q, V; X_2 is F, H, L, N, Q, R, S, T or V; X_3 is C, F, I, L, M, R, S, V or W; X_4 is any of the 20 genetically coded L-amino acids; X_5 is A, D, E, G, K, M, Q, R, S, T, V or Y; X_6 is C, F, G, L, M, S, V, W or Y; and X_7 is C, G, I, K, L, M, N, R or V.

In a preferred embodiment the core peptide comprises a sequence of amino acids --(SEQ ID NO:3)--:

X_1 G X_2 X_3 X_4 X_5 X_6 W X_7

where X_1 is L, M, P, Q, or V; X_2 is F, R, S, or T; X_3 is F, L, V, or W; X_4 is A, K, L, M, R, S, V, or T; X_5 is A, E, G, K, M, Q, R, S, or T; X_6 is C, I, K, L, M or V; and each X_7 residue is independently selected from any of the 20 genetically coded L-amino acids, their stereoisomeric D-amino acids; and non-natural amino acids. Preferably, each X_7 residue is independently selected from any of the 20 genetically coded L-amino acids and their stereoisomeric D-amino acids. In a preferred embodiment --(SEQ ID NO:4)--, X_1 is P; X_2 is T; X_3 is L; X_4 is R; X_5 is E or Q; and X_6 is I or L.

More preferably, the core peptide comprises a sequence of amino acids -- SEQ ID NO:5 --:

X_1 X_2 G X_3 X_4 X_5 X_6 X_7 W X_8

where X_1 is A, C, E, G, I, L , M, P, R, Q, S, T, or V; and X_2 is A, C, D, E, K, L, Q, R, S, T, or V. More preferably, X_1 is A or I; and X_2 is D, E, or K.

Particularly preferred peptides include --(SEQ ID NOS 6-13, RESPECTIVELY)--: G G D A D G P T L R E W I S F C G G; G N A D G P T L R Q W L E G R R P K N; G G D A D G P T L R E W I S F C G G K; T I K G P T L P Q W L K S R E H T S; S I E G P T L R E W L T S R T P H S; L A I E G P T L R Q W L H G N G

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R D T; C A L S E T L R E W I S F J; M I E S E T L R, W L A

A P A.

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In further embodiments of the invention, preferred peptides for use in this invention include peptides having a core structure comprising sequence of amino acids: sequence of amino acids -- SEQ ID NO:14 --:

C X_1 X_2 X_3 X_4 X_5 X_6

where X_1 is F, K, L, N, Q, R, S, T or V; X_2 is C, F, I, L, M, R, S or V; X_3 is any of the 20 genetically coded L-amino acids; X_4 is A, D, E, G, S, V or Y; X_5 is C, F, G, L, M, S, V, W or Y; and X_6 is C, G, I, K, L, M, N, P or V. In a more preferred embodiment, X_1 is A, E, G, H, K, L, M, P, Q, R, S, T, or W. In a further embodiment, X_2 is S or T; X_3 is L or R; X_4 is R; X_5 is D, E, or G; X_6 is F, L, or W; and X_7 is I, K, L, R, or V. Particularly preferred peptides include --(SEQ ID NO:15)--: G G C T L R E W L H G G F C G G.

In a further embodiment, preferred peptides for use in this invention include peptides having a structure comprising a sequence of amino acids --(SEQ ID NO:16)--:

X_1 C X_2 X_3 X_4 X_5 X_6 X_7

where X_1 is F, K, L, N, Q, R, S, T or V; X_2 is C, F, I, L, M, R, S, V or W; X_3 is any of the 20 genetically coded L-amino acids; X_4 is A, D, E, G, K, M, Q, R, S, T, V or Y; X_5 is C, F, G, L, M, S, V, W or Y; X_6 is C, G, I, K, L, M, N, R or V; and X_7 is any of the 20 genetically coded L-amino acids. In some embodiments, X_1 is preferably G, S, Y, or R.

Peptides and peptidomimetics having an IC_{50} of greater than about 100 mM lack sufficient binding to permit use in either the diagnostic or therapeutic aspects of this invention. Preferably, for diagnostic purposes, the peptides and peptidomimetics have an IC_{50} of about 2 mM or less and, for pharmaceutical purposes, the peptides and peptidomimetics have an IC_{50} of about 100 uM or less.

The binding peptide sequence also provides a means to determine the minimum size of a TPOR binding compound of

the invention. Using the "encoded synthetic library" ESL system or the "very large scale immobilized polymer synthesis"

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intraperitoneally was administered on day 1. AF11513 1 mg/kg, ip was given on Days 1-5. These results show the peptides of the invention can ameliorate thrombocytopenia in a mouse model.

In addition, certain peptides of the present invention can be dimerized or oligomerized, thereby increasing the affinity and/or activity of the compounds. To investigate the effect that peptide dimerization/oligomerization has on TPO mimetic potency in cell proliferation assays, a C-terminally biotinylated analog of the peptide --(SEQ ID NO:6)-- G G C A D G P T L R E W I S F C G G was synthesized --(SEQ ID NO:8)-- (G G C A D G P T L R E W I S F C G G K (Biotin)). The peptide was preincubated with streptavidin in serum-free HEPES-buffered RPMI at a 4:1 molar ratio. The complex was tested for stimulation of cell proliferation of TPO-R transfected Ba/F3 cells, as above, alongside free biotinylated peptide and the unbiotinylated parental peptide. Figure 2A shows the results of the assay for the complexed biotinylated peptide (AF 12885 with streptavidin (SA)) for both the transfected and parental cell lines. Figure 2B shows the results of the assay for the free biotinylated peptide (AF 12185) for both the transfected and parental cell lines. Figure 2C shows the results of the assay for streptavidin alone for both the transfected and parental cell lines. These figures illustrate that the pre-formed complex was approximately 10 times as potent as the free peptide.

The specificity of the binding and activity of the peptides of the invention was also examined by studying the cross reactivity of the peptides for the erythropoietin receptor (EPO-R). The EPO-R is also a member of the haematopoietin growth factor receptor family, as is TPO-R. The peptides of the invention, as well as TPO, EPO, and a known EPO-binding peptide, were examined in a cell proliferation assay using an EPO-dependent cell line. This

assay utilised FDCP-1, a growth factor dependent murine multi-potential primitive haematopoietic progenitor cell line

REPLACEMENT PAGE

EXAMPLE 4

"PEPTIDES ON PLASMIDS"

The p8141 vector is used for library construction and is shown in Figure 4. Three oligonucleotide sequences -- SEQ ID NOS 169-171, respectively -- are needed for library construction: ON-829 (5' ACG ACG TGG GG ; ON-830 3' TTA CTT AGT TA) and a library specific oligonucleotide of interest (5' GA GGT GGT (NNK)$_n$ TAA TTA AGT AAA GG , where (NNK)$_n$ denotes a random region of the desired length and sequence. The oligonucleotides can be 5' phosphorylated chemically during synthesis or after purification with polynucleotide kinase. They are then annealed at a 1:1:1 molar ratio and ligated to the vector.

The strain of *E. coli* which is preferably used for panning has the genotype: *Δ(srl-recA) endA1 nupG lon-11 sulA1 hsdR17 Δ(ompT-fepC)266 ΔclpA319::kan ΔlacI lac ZU118* which can be prepared from an *E. coli* strain from the *E. coli* Genetic Stock Center at Yale University (*E. coli* b/r, stock center designation CGSC:6573) with genotype *lon-11 sulA1*. The above *E. coli* strain is prepared for use in electroporation as described by Dower et al. Nucleic Acids Res. **16:6127** (1988), except that 10% glycerol is used for all wash steps. The cells are tested for efficiency using 1 pg of a Bluescript plasmid (Stratagene). These cells are used for growth of the original library and for amplification of the enriched population after each round of panning.

Peptides on plasmids are released from cells for panning by gentle enzymatic digestion of the cell wall using lysozyme. After pelleting of the cell debris, the crude lysate can be used directly on most receptors. If some additional purification of the plasmid complexes is needed, a gel filtration column can be used to remove many of the low molecular weight contaminants in the crude lysate.

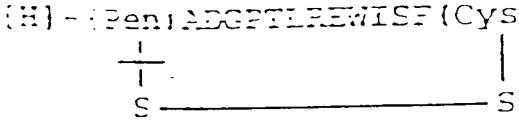
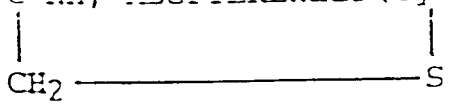
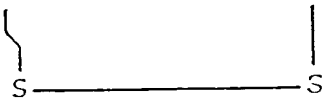
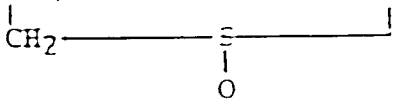
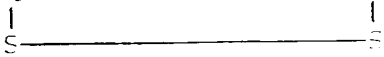
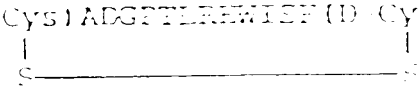
Panning is carried out in a buffer HEPES at a lower salt concentration than most physiological buffers. The

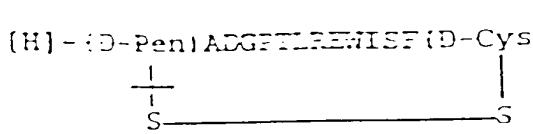
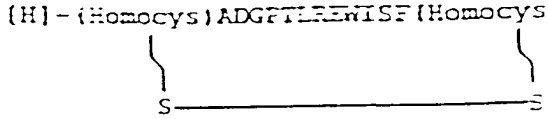
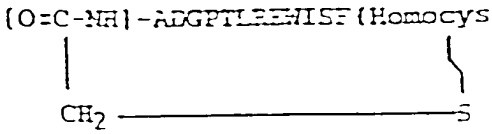
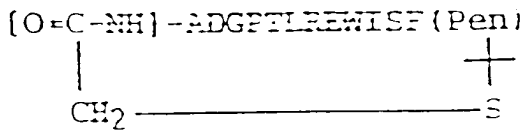
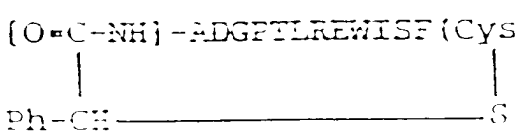
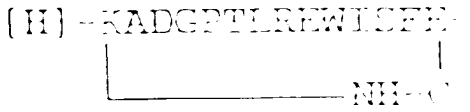
REPLACEMENT PAGE

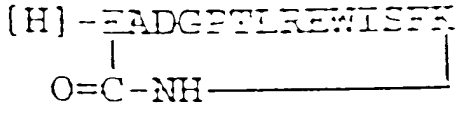
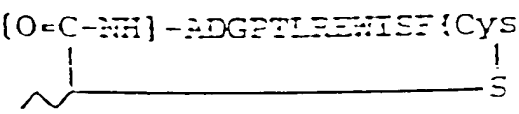
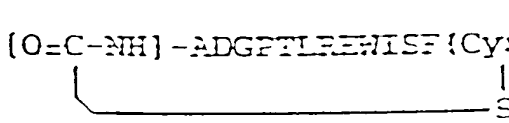
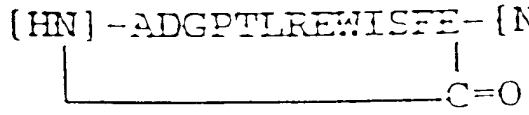
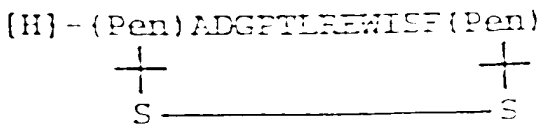
Panning is carried out in a buffer HEPL of a lower salt concentration than most physiological buffers. The

REPLACEMENT PAGE

TABLE 4

Structure	EC50(nM) Proliferation	EC50(nM) Microonyx	IC50(nM)
{H}-{Pen}ADGPTLREWISF{Cys}-{NH2}--(SEQ ID NO:172)-- 	++	++	++
{O=C-NH}-ADGPTLREWISF{Cys}-{NH2}--(SEQ ID NO:173)-- 	++	++	++
{H}-{Homocys}ADGPTLREWISF{Cys}-{NH2}--(SEQ ID NO:174)-- 	++	++	ND
{O=C-N}-ADGPTLREWISF-{Cys}-{NH2}--(SEQ ID NO:173)-- 	+	+	+
{H}-{D-Cys}ADGPTLREWISF{D-Cys}-{NH2}--(SEQ ID NO:175)-- 	+	+	ND
{H}-{Cys}ADGPTLREWISF{D-Cys}-{NH2}--(SEQ ID NO:176)-- 	+	+	++

Structure	EC50(nM) Proliferation	EC50(nM) Microenvs	IC50(nM)
			
			
			
			
			
			

Structure	EC50(nM) Proliferation	EC50(nM) Microonys.	IC50(nM)
 --(SEQ ID NO:182)--	+	+	ND
 --(SEQ ID NO:173)--	+ +	+	ND
 --(SEQ ID NO:173)--	+ +	+	ND
 --(SEQ ID NO:183)--	+	+	+
 --(SEQ ID NO:184)--	+	+	ND

EXAMPLE 7

In this example amino acid substitutes at positions B, D, E, I, S, or F in the cyclized compound -- SE_2 in NO:12 --

C A D G P T L R E W I S F C

were assayed for EC_{50} and IC_{50} values as described above. Microphysiometer results are given in parentheses. The results are summarized in Table 5 below.

TABLE 5

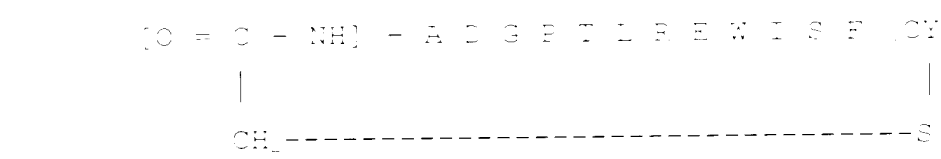
--(SEQ ID NO:12)--

CADGPTLREWISFC

Substitution	EC50 (nM) Cell Prolif.	IC50 (nM)
E - Q	++ (+)	++
D - A	+ (+)	++
I - A	+- (+)	+-
S - A	++ (++)	++
S - D-Ala	+	+-
S - Sar	+-	++
S - Aib	++ (+)	++
S - D-Ser	++	++
S - Nva	++ (++)	++
S - Abu	++	++
S - (N-Me-Ala)	+-	+
S - (N-Me-Val)	+	+-
S - (N-Me-Ala)	+-	+-
S - (Nor-Leu)	++	++
S - (t-Bu-Gly)	+	++
S - [N-Me-Ser(Bzl)]		+

EXAMPLE 8

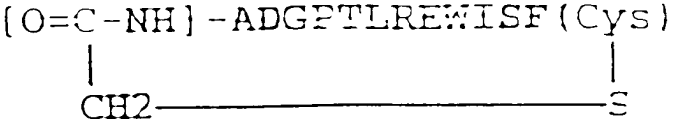
In this example, amino acid substitutions in the compound -- SEQ ID NO:173 --



were evaluated at positions D, S, or F as indicated in Table 6 below. EC_{50} and IC_{50} values were calculated as described above. Microphysiometer results are in parentheses.

TABLE 6

--(SEQ ID NO:173)--



Substitution	EC50 (nM) Cell Prolif.	IC50 (nM)
D - E	(+)	ND
free acid form	++ (+)	ND
C-term. Gly addition	++	++
S - Abu	++(++)	ND
F - DiPh-Ala	(++)	++
S,F - Abu, DiPh-Ala	+(+)	++

EXAMPLE 9

In this example EC_{50} and IC_{50} values were calculated as described above for the dimer compounds listed in Table 7 below. The cyclized monomer -- SEQ ID NO:12 --

C A D G P T L R E W I S F C

is included as a comparison.

The compounds of Table 8 were inactive at the maximum concentration tested of 10μm.

In Table 9, EC_{50} and IC_{50} values determined as described above for cyclized and dimerized variants of --(SEQ ID NO:193)--

I E G P T L R Q W L A A R A are compared.

In Table 10, truncations of the dimer --(SEQ ID NOS 17 & 18, respectively)--

(H) - I E G P T L R Q W L A A R A

(H) - I E G P T L R Q W L A A R A (βala) K - (NH_2)

are compared. EC_{50} and IC_{50} values were calculated as described above. Microphysiometer results are given in parentheses.

TABLE 7

	EC50 (nM) Microphys.	EC50 (nM) Prolif.	IC50 (nM)
{Br-C(=O)-NH}-ADGPTLREWISFC-{NH_2} --(SEQ ID NO:173)--	++	++	++
{Br-C(=O)-NH}-ADGPTLREWISFC-{NH_2} --(SEQ ID NO:173)--			
{H}-EEGPTLRQWLAARA --(SEQ ID NO:17)--	++	++	--
{H}-EEGPTLRQWLAARA(β-Ala)K-{NH_2} --(SEQ ID NO:18)--			
{H}-CEEGPTLRQWLAARA-{NH_2} --(SEQ ID NO:185)--	++	++	--
{H}-CEEGPTLRQWLAARA-{NH_2} --(SEQ ID NO:185)--			
{H}-CADGPTLREWISF-{NH_2} --(SEQ ID NO:186)--	++	++	--
{H}-CADGPTLREWISF-{NH_2} --(SEQ ID NO:186)--			
{H}-SVQCGPTLRQWLAARNHLS-{NH_2} --(SEQ ID NO:187)--	++	++	--
{H}-SVQCGPTLRQWLAARNHLS-{NH_2} --(SEQ ID NO:187)--			
{H}-HVGPTLRSGC-{NH2} --(SEQ ID NO:188)--	ND	+	--
{H}-HVGPTLRSGC-{NH2} --(SEQ ID NO:188)--			

	EC50 (nM) Microphys.	EC50 (nM) Prolif.	IC50 (nM)
CADGPTLREWISFC --(SEQ ID NO:12)--	++	++	++
[Ac]-ADGPTLREWISFC --(SEQ ID NO:173)--	ND	++	++
[Ac]-ADGPTLREWISFC --(SEQ ID NO:173)--			
ADGPTLREWISFC --(SEQ ID NO:173)--	++	++	++
ADGPTLREWISFC --(SEQ ID NO:173)--			
[Ac]-EGPTLREWISFC --(SEQ ID NO:189)--	++	++	++
[Ac]-EGPTLREWISFC --(SEQ ID NO:189)--			
[Ac]-GPTLREWISFC --(SEQ ID NO:190)--	ND	++	++
[Ac]-GPTLREWISFC --(SEQ ID NO:190)--			
GPTLREWISFC --(SEQ ID NO:190)--	++	++	+
GPTLREWISFC --(SEQ ID NO:190)--			
[Ac]-PTLREWISFC --(SEQ ID NO:191)--	ND	++	++
[Ac]-PTLREWISFC --(SEQ ID NO:191)--			
PTLREWISFC --(SEQ ID NO:191)--	++	++	+
PTLREWISFC --(SEQ ID NO:191)--			
[Ac]-TLREWISFC --(SEQ ID NO:192)--	+	+	+
[Ac]-TLREWISFC --(SEQ ID NO:192)--			
TLREWISFC --(SEQ ID NO:192)--	++	+	+
TLREWISFC --(SEQ ID NO:192)--			

TABLE 8

--(SEQ ID NOS 205-222, respectively)--

{H}-CTRAQFLKGC-{NH_2}

{H}-CHINQLRSIC-{NH_2}

{H}-CNRSQLLAAC-{NH_2}

{H}-CTSTQWLLAC-{NH_2}

{H}-CQRADLINFC-{NH_2}

{H}-CILSEFLAGQQC-{NH_2}

{H}-CTFQVWKLARNC-{NH_2}

{H}-CTLGQWLQMEEC-{NH_2}

{H}-CLTGPFVTQWLYEC-{NH_2}

{H}-CTLREFLDPTTAVC-{NH_2}

{H}-CGTEGPTLSTWLDC-{NH_2}

{H}-CELVGPSLMSWLTC-{NH_2}

{H}-CSLKEFLNSGLMQC-{NH_2}

{H}-CTLAEFLASGVEQC-{NH_2}

{H}-CTLKEWLVSHEVMC-{NH_2}

TABLE 9

	EC50 (nM) Microphys.	EC50 (nM) Prolif.	IC50 (nM)
[H]-IEGPTLRQWLAARA-[NH_2] --(SEQ ID NO:193)--	N.D.	++	++
[H]-CIEGPTLRQWLAARAC-[NH_2] --(SEQ ID NO:194)--	N.D.	++	++
[H]-IEGPTLRQWLAARA --(SEQ ID NO:17)--	++	++	++
[H]-IEGPTLRQWLAARA(ß-Ala)K-[NH_2] --(SEQ ID NO:18)--			
[H]-CIEGPTLRQWLAARA-[NH_2] --(SEQ ID NO:185)--	++	++	++
[H]-CIEGPTLRQWLAARA-[NH_2] --(SEQ ID NO:185)--			

TABLE 10

(H)-IEGPTLRQWLAARA
|
(H)-IEGPTLRQWLAARA(β-Ala)K-(NH_2) --(SEQ ID NOS 17 & 18)--

Sequence	EC50 (nM) Cell Prolif.	IC50 (nM)
(Ac)-IEGPTLRQWLAARA | (Ac)-IEGPTLRQWLAARA-βA-K(NH_2) --(SEQ ID NOS 17 & 18)--	++	ND
(H)-IEGPTLRQWLAAR | (H)-IEGPTLRQWLAAR-βA-K(NH_2) --(SEQ ID NOS 195 & 196)--	++	ND
(H)-IEGPTLRQWLAA | (H)-IEGPTLRQWLAA-βA-K(NH_2) --(SEQ ID NOS 197 & 198)--	++(++)	ND
(Ac)-EGPTLRQWLAARA | (Ac)-EGPTLRQWLAARA-βA-K(NH_2) --(SEQ ID NOS 199 & 200)--	ND	ND
(H)-EGPTLRQWLAARA | (H)-EGPTLRQWLAARA-βA-K(NH_2) --(SEQ ID NOS 199 & 200)--	++	ND
(H)-EGPTLRQWLAAR | (H)-EGPTLRQWLAAR-βA-K(NH_2) --(SEQ ID NOS 201 & 202)--	++(++)	ND
(Ac)-EGPTLRQWLAA | (Ac)-EGPTLRQWLAA-βA-K(NH_2) --(SEQ ID NOS 203 & 204)--	+	ND
(H)-EGPTLRQWLAA | (H)-EGPTLRQWLAA-βA-K(NH_2) --(SEQ ID NOS 203 & 204)--	+	ND

EXAMPLE 10

In this example various substitutions were introduced at positions G, P, and W in the cyclized compound -- SEQ ID NO:11 --

[H] - C A D G P T L R E W I S F C - [NH_2].

Table 11 lists examples of the substituted compounds that show TPO agonist activity. The substitutions abbreviated in the table are as follows:

TABLE 11

[H] - C A D G P T L R E W I S F C - [NH_2]		
G	**P**	**W**
Sar	Hyp(OBn)	Nal
Sar	Hyp(OBn)	Nal
Gly	Pro	Trp
Gly	Pro	Trp
Sar	Hyp(OBn)	Nal
Gaba	Pro	Trp
Cpr-Gly	Pro	Trp
Sar	Hyp(OBn)	Nal
Gly	Pro	Trp
Gly	Pro	Nal
Sar	Pro	Trp
Cpr-Gly	L-Tic	Nal
Gly	D-Tic	D-Trp
Cpr-Gly	D-Tic	Trp
Gaba	Hyp(OBn)	Trp